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Starvation triggers $A\beta_{42}$ generation from human umbilical vascular endothelial cells

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ABSTRACT

Cerebral amyloid angiopathy is a common feature in Alzheimer's disease (AD), which is characterized by amyloid deposit around brain vessels including capillaries. The origin of the amyloid protein of CAA remains controversial. In our work, we provide data to show that primary umbilical vein endothelial cells (HUVEC) harbor APP processing secretases and can produce $A\beta_{42}$ under starvation. Starvation can increase the secretion of $A\beta_{42}$ by altering the expression of β-secretases (BACE1) and γ-secretases (APH and PEN2). This process is regulated by macroautophagy. Suppression of macroautophagy induction by 3MA further increased the level of $A\beta_{42}$ produced under starvation in HUVECs. These results suggest that starvation-induced $A\beta_{42}$ secretion might contribute to the formation of CAA and hence vascular degeneration in AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized with progressive decline of memory, cognitive and visuospatial function [1]. Recently, many evidences indicated that vascular system also contributed to the pathogenesis of AD [2,3]. Epidemiological data showed that vascular factors increased the incidence of AD, for example diabetes, hypertension, atherosclerosis and stroke [2]. Neuroimaging technique such as single-photon emission computed tomography (SPECT) detected reduced regional cerebral blood flow (CBF) in mild cognitive impairment (MCI) patient which is a preclinical stage of AD [4]. Postmortem studies also found that vascular changes such as reduced microvascular density, dilated vessel lumen and cerebral amyloid angiopathy (CAA) were common in AD brain [5].

CAA is a pathologic feature of AD which is defined as amyloid beta (Aβ) deposit around the brain vessels [6]. Patients suffered from CAA have vessel dysfunction resulting in cerebral infarction and more commonly cerebral hemorrhages. It has been shown that CAA interacted with other neuropathological changes and contributed to cognitive decline and dementia in AD patients [7–9]. Some even proposed that CAA played a direct role in the pathogenesis of AD [10].

Aβ is the main component of senile plaque and CAA. In neuron, Aβ is produced from APP through sequential cleavage by β-secretase (BACE1) and γ-secretase complex including (PS1, PS2, nicastrin, PEN2 and APH1). Starvation is a harmful condition that could cause nutrient, glucose and energy deficiency. Result from epidemiological study showed that starvation increased the incidence of sporadic Alzheimer's disease [11]. Data from APP transgenic mouse and cell models study also indicated that starvation could trigger the amyloidogenic pathway, possibly by facilitating the β- and γ-cleavage of APP through increasing BACE1 and γ-secretases expression, and macroautophagy [12–14]. However, whether starvations is also implicated in the Aβ generation from vascular origin and hence contributes to the CAA formation has never been well studied yet.

In the present study, we tested that (1) whether starvation could trigger the $A\beta_{42}$ from HUVEC and if so (2) what the possible mechanism under this; (3) whether macroautophagy is involved in; (4) what role of macroautophagy played in this process. From our work, we found that starvation triggered $A\beta_{42}$ generation in HUVEC.

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Moreover, starvation could activate amyloidogenic processing of APP by regulating BACE1 and γ-secretase (PEN2 and APH). However, macroautophagy was not a trigger factor in our model and inhibiting it could further increased excellular $A\beta_{42}$ level under starvation. In one word, our data suggests that starvation-induced generation of $A\beta_{42}$ from endothelium might contribute to the CAA formation and vessel degeneration in AD. Macroautophagy perhaps served to prevent $A\beta_{42}$ production in HUVEC induced by starvation.

2. Materials and methods

2.1. Cell cultures

Primary human umbilic vascular endothelial cell (HUVEC) was purchased from cascade company (C-023-5C, oragon, USA). HEK293T was purchased from Chinese science institute; HEK293T was cultured in DMEM with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. HUVEC was cultured in cascade medium 200 (M-200-500) with LSGS (S-003-10) and used no more than ten passages. All other media, culture products and chemicals were purchased from Gibco Company (Paisley, UK).

2.2. Macroautophagic assay

To testify whether starvation could induce macroautophagy in HUVEC, EBSS (eagles balanced salt solution) (Gibco, 14155) was used to treat HUVEC to induce starvation (Gibco, 14155) [15,16]. Macroautophagy was measured by western blot analysis of the level of LC3 (1:500, Sigma, L7543), immunofluorescent LC3 staining (1:50) and electron microscopy. Autophagic inhibitor 3-MA was purchased from Sigma (M9281, R0395). The concentration of 3-MA (1 mM) was determined by the sub-lethal and highest changes of autophagic marker – LC3 protein expression. Ammonium chloride (NH_4Cl, 20 mM) was used as an agent to prevent autophagosome–lysosome fusion. They were added into EBSS without serum 4 h before harvest.

2.3. Electron microscopy

Autophagic vacuoles in HUVEC under control and starvation were measured by EM in the following method. Cultured HUVEC intended for EM was removed cultured media, washing with 37 °C supplement free LCSC, harvested in 1.5 ml tubes and centrifuge at 1500×*g* for 5 min. The precipitation was fixed in 2% glutaraldehyde for 2 h at 4 °C in phosphate-buffered saline (PBS). After washing in PBS, the cells were postfixed in 1% osmic acid (OsO_4) at 4 °C for another 2 h and subsequently rapidly dehydrated through a graded series of ethanol (30–100%). The samples were then infiltrated in a 1:2 Epon Resin 618/PO mixture overnight and subsequently embedded in Epon Resin 618. Ultrathin sections were stained with uranyl acetate followed by lead citrate and observed using a Philips CM120 transmission electron microscope.

2.4. Immunofluorescent staining

To testify whether HUVEC harbored the secretases required for APP processing, immunofluorescent staining was used to probe for the different β and γ secretases as followed. Cell was cultured on the 24-well plate with coverslip under it. After treatment, cell-mounted coverslip were fixed in 4% paraformaldehyade, blocking with 10% BSA and sequentially incubated at room temperature with intervening rinses, 10% BSA with 0.2% Triton X-100, primary antibody BACE1 (1:100, Calbiochem), PS1 (1:100, Sigma), nicastrin (1:100, Sigma), APH (1:100, Sigma), PEN2 (1:100, Zymed), ADAM17 (1:100, Millipore, CA) and LC3 (1:200, Sigma) overnight at 4 °C. Alexa488 – conjugated secondary antibodies (1:800, invitrogen) were used to stain bound primary antibodies at routine temperature for 1hr after rinsing. Image capture was made with the Olympus DP controller system link to the Olympus IX70 (Olympus 1.1.1.65).

2.5. Western blot

The level of macroautophagic protein LC3, β and γ secretases under control, after starvation was measured by immunoblotting as followed. Cells were harvested with a lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 0.5% Tween 20 with protease inhibitor cocktail (Roche) on ice for 30 min. Protein concentration was determined by Bio-Rad BCA protein assay (Bio-Rad, CA). Protein sample (50 μg) were loaded in 7.5–17.5% Tris–glycine gels and electrophoresed at 80 mA for 2–3 h and transferred onto 0.2 μm or 0.45 μm nitrocellulose membrane (Millipore) at 90 mV for 2–3 h depending on the targeted protein. Membranes were incubated at 4 °C overnight with primary antibody:anti-LC3 (1:5000, Sigma), anti-PS1 (1:10 000, Sigma), anti-PEN2 (1:250, Zymed), anti-nicastrin (1:1000, Sigma), anti-aph1(1:1000, Sigma), anti-BACE1 (1:100, Calbiochem). Secondly antibody: anti-mouse (1:5000, Sigma), anti-rabbit (1:5000, Sigma) was incubated for 1 h at room temperature. Bands were visualized by chemiluminescence substrate (HyGLO, E2500).

2.6. $A\beta_{42}$ quantification

The level of $A\beta_{42}$ generated from HUVEC after starvation and 3MA treatment was measured by enzyme-linked immunosorbent assay kit for $A\beta_{42}$ (ELISA) (ExCell Bio, EH025). After starvation, medium was collected. The extracellular level of $A\beta_{42}$ was measured according to the manufacturer's protocol.

2.7. β-Secretase activity quantification

The β-secretase activities under control and starvation were measured by enzyme-linked immunosorbent assay kit (Biovision, K360-100) according to the manufacturer's protocol.

2.8. Statistical analysis

Data are expressed as the means ± S.E.M. The value was calculated from the number of experiments indicated in corresponding figures. One way ANOVA test was performed to compare the difference between groups with SPSS analysis software. The level of $P < 0.05$ was regarded as statistical significance.

3. Results

3.1. Starvation triggers production of $A\beta_{42}$ from HUVEC

Starvation has been shown to trigger the amyloidogenic pathway in neuron [12]. However, this has never been tested in HUVEC. We measured the extracellular level of $A\beta_{42}$ after starvation in HUVEC. Using EBSS, we found that starvation could trigger the production of $A\beta_{42}$ from HUVEC. We observed that the extracellular level of $A\beta_{42}$ in HUVEC increased in 2 h of starvation and this reached to nearly 2-fold in 4 h (2.31 ± 0.49%, $P = 0.007$), and then decreased in 12 h (Fig. 1A).

3.2. Detection of secretases in amyloidogenic pathway in HUVEC

APP was cleaved through α-secretase (ADAM 17, ADAM 10) in non-amyloidogenic pathway and through β-secretase (BACE1)

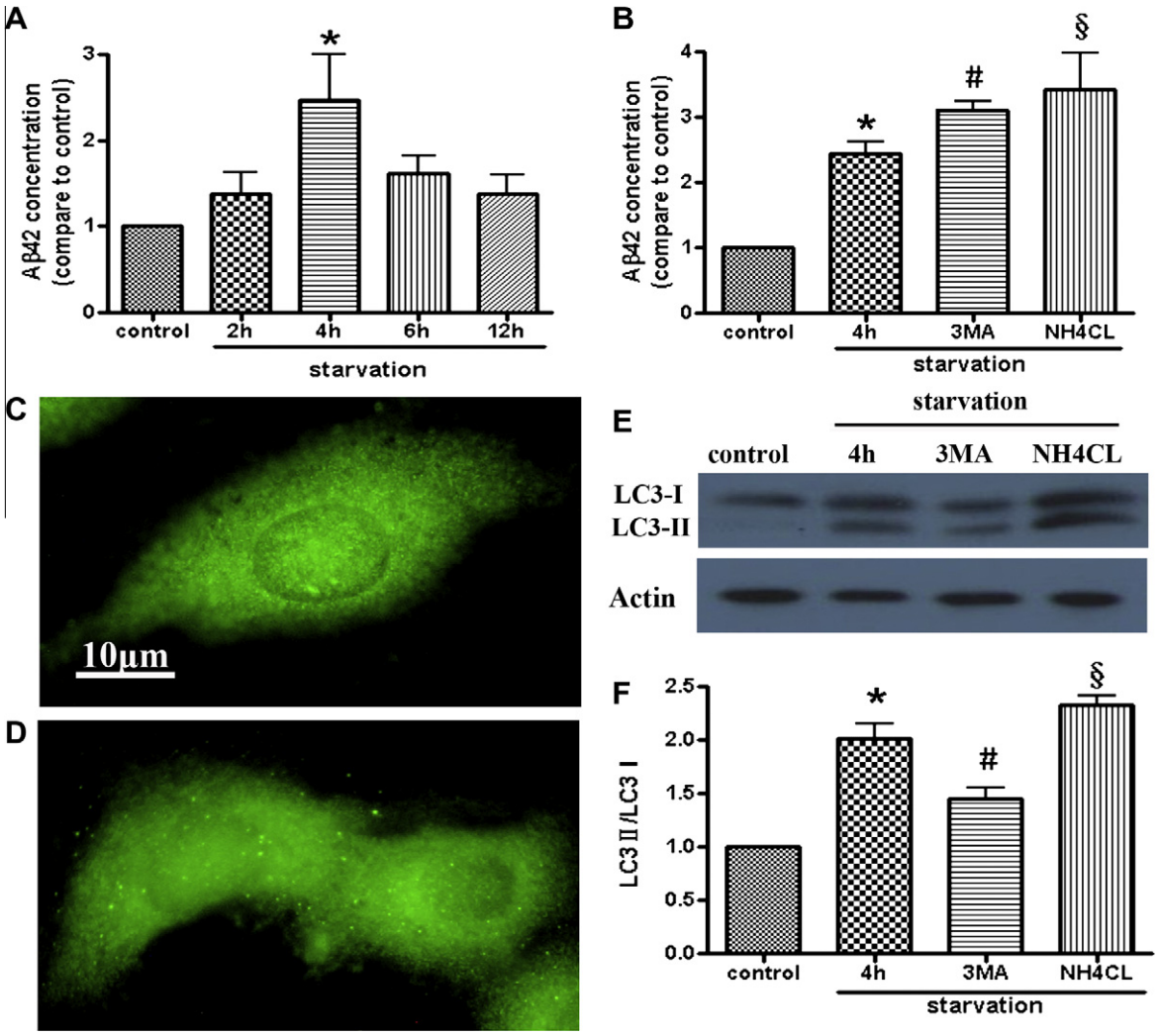

Fig. 1. Macroautophagy was involved in starvation-induced $A\beta_{42}$ secretion in human umbilical vein endothelial cells. Secretion of $A\beta_{42}$ by HUVEC after starvation was measured and normalized against control as in time-course (A) and macroautophagy inhibition by 3MA and NH_4Cl (B). Immunofluorescent staining for LC3 was depicted by microscopy in control HUVEC (C) and starvation-treated HUVEC (D). Starvation-treated HUVEC showed punctuate LC3 staining representative of AV membranes. Western blot analyses of LC3 (E) confirmed the macroautophagy induction under starvation and manipulating of macroautophagy with 3MA and NH_4Cl changed LC3 expression representative of macroautophagy status. The ratios of LC3-II to LC3-I were measured and normalized against control (F). Data are means ± S.E.M. values of 3–6 independent experiments; $^*P < 0.05$.

and γ-secretases (PS1, PS2, nicastrin, APH, PEN2) in amyloidogenic pathway in neuron. Although Selko et al. has shown that pathway existed in HUVEC which could generate amyloid fragments, it has never been tested whether HUVEC harbor the machinery for the amyloidogenic processing. So we probed the HUVEC with antibody against ADAM17, BACE1, PS1, nicastrin, APH, PEN2 in order to demonstrate that all the APP processing proteins were existed in HUVEC. The immunofluorescent staining confirmed that HUVEC harbored the β-secretase and γ-secretases needed for APP cleavage which suggested that HUVEC has the potential to generate $A\beta_{42}$ under starvation (Fig. 2A, left column).

3.3. Starvation triggered $A\beta_{42}$ production from HUVEC by increasing the expression of BACE1 and γ-secretase (APH and PEN2)

Amyloidogenic pathway was mediated by β-secretase and γ-secretase cleavage of APP in neuron. Starvation has been implicated in amyloid production by increasing BACE1 and γ-secretase in vitro and in vivo models. Accordingly, we studied whether β-secretase and γ-secretase was involved in the changes of extracellular $A\beta_{42}$ level triggered by starvation in HUVEC.

We firstly tested the BACE1 expression under starvation. Immunofluorescent showed that BACE1 staining was increased (Fig. 2A, right column) and further immunoblot staining of BACE1 confirmed that it was elevated after starvation for 2 h and reached the significant level at 4 h (1.87 ± 0.19%, $P = 0.039$) (Fig. 2C).

Then we further tested the expression of γ-secretases. Normally γ-secretases complex include different proteins named PS1, PS2, nicastrin, APH and PEN2. The results showed that the expression of PEN2 (1.75 ± 0.1%, $P = 0.003$) and APH (1.72 ± 0.13%, $P = 0.034$) increased after starvation both in immunofluorescent (Fig. 2A, right column) and immunoblot staining (Fig. 2D and E), but the level of nicastrin and PS1 remained constantly (data not shown). The same experiments were repeated in HEK293 (data not shown). The β-secretase activities were also increased after starvation and reached the statistical significance at 4 h (1.50 ± 0.03%, $P = 0.002$) (Fig. 2F).

3.4. Starvation induces macroautophagy in HUVEC

Macroautophagy was already shown involved in APP processing in neuron. However, it was unknown whether macroautophagy also played a role in starvation-induced amyloidogenic pathway. In our study, we confirmed that starvation could induce

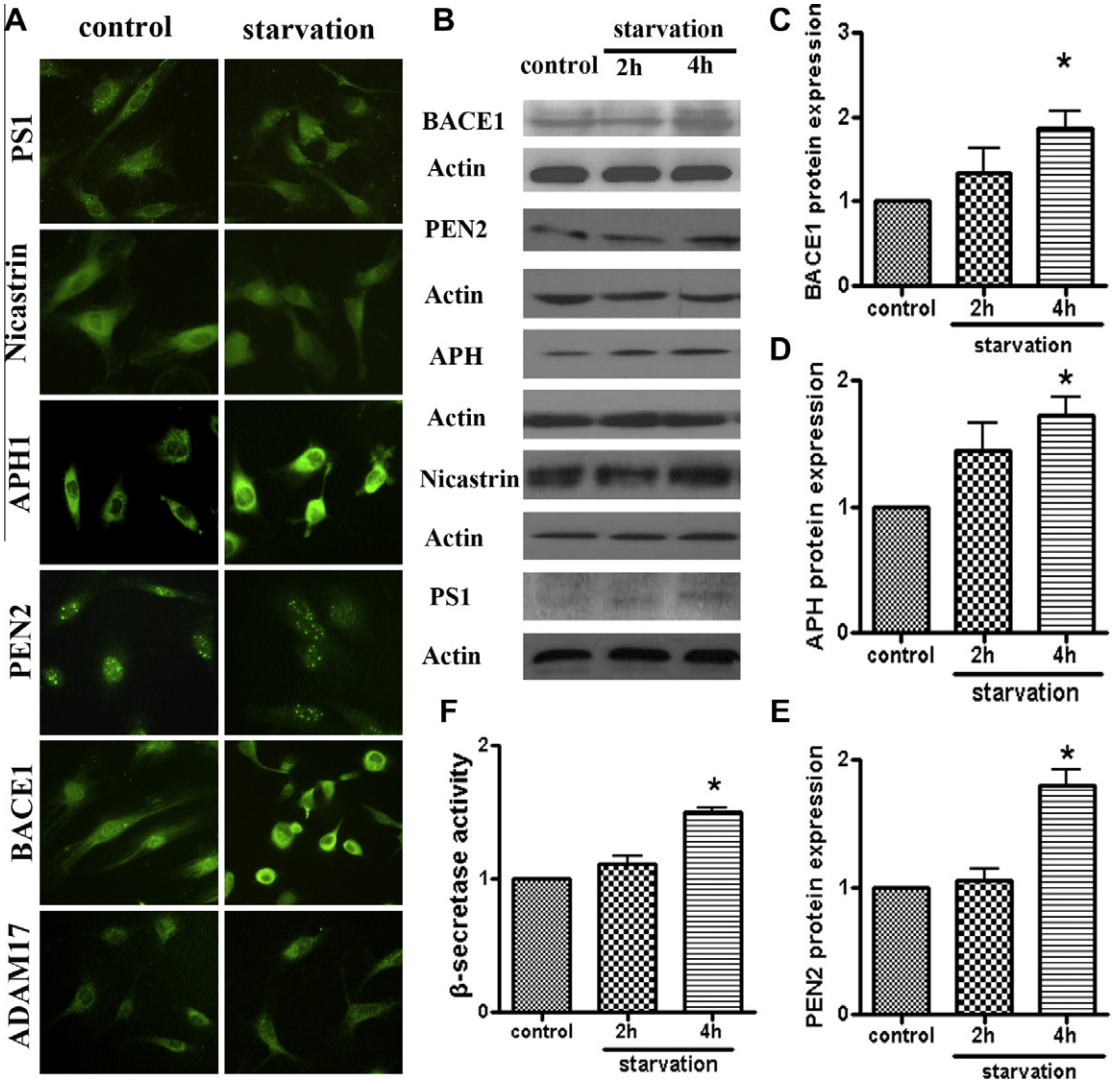

Fig. 2. Detection of APP processing enzymes in human umbilical vein endothelial cell. The presence of α-secretases (ADAM17), β-secretase (BACE1) and γ-secretase (PS1, Nicastrin, APH and PEN2) were analyzed by immunofluorescence staining in HUVEC under normal condition (A; left panel) and starvation for 2 and 4 h (A; right panel). Inhibition macroautophagy increase the amyloidogenic APP pathway in HUVECs. Western blot of BACE1, PEN2, APH, Nicastrin and PS1 were shown after starvation (B). Average band density of BACE1 (C), PEN2 (D), APH (E) was normalized against actin. β-Secretase activities were measured and normalized against control (F). Data are means ± S.E.M. values of 3–6 independent experiments; $^{*}P < 0.05$.

macroautophagy pathway in HUVEC shown by changes of LC3 staining and autophagic vacuoles in EM (electron microscopy). Immunofluorescent staining revealed punctuate LC3 representing AV (autophagic vacuoles) membrane in HUVEC after starvation (Fig. 1C and D). Western blot showed the level of LC3||/LC3| increased after starvation (2.02 ± 0.13%, $P = 0.00$) and decreased after 3MA treatment (1.45 ± 0.10%, $P = 0.008$) by inhibiting the induction of macroautophagy under starvation. As described before, NH_4Cl treatment, an agent neutralizes acidic compartments, prevented autophagosome–lysosome fusion and thus increased the level of LC3||/LC3| (2.32 ± 0.09%, $P = 0.044$) probably by blocking the LC3 degradation (Fig. 1E and F). EM also revealed the buildup of autophagic vacuoles (AV) structures, such as autophagosome and multilamellar bodies (Fig. 3), further demonstrating the macroautophagic induction in HUVEC after starvation.

3.5. Inhibition of macroautophagy increased starvation-triggered $A\beta_{42}$ production from HUVEC

Macroautophagy could not only facilitate the APP cleavage but also promote Aβ degradation. What role that macroautophagy played in starvation triggered $A\beta_{42}$ production has been never testified. Our work found that suppressing the induction of macroautophagy with 3MA enhanced the increased secretion of $A\beta_{42}$ (3.18 ± 0.12%, $P = 0.013$) (Fig. 1B). Ammonium chloride (NH_4Cl) further increased extracellular level of $A\beta_{42}$ even more significant than 3MA (3.41 ± 0.58%, $P = 0.008$) (Fig. 1B). This suggested that macroautophagy might play a protective role in the process of starvation-triggered $A\beta_{42}$ production from HUVEC.

4. Discussion

So far, different sources of Aβ on the CAA have been proposed: (1) accumulation of neuronal produced Aβ drainage through the perivascular system [17,18]; (2) production of Aβ from the vascular smooth muscle cells [19–21]; (3) production of Aβ from the pericyte around the vessel [10]. Neuronal contribution to the CAA was strongly supported because transgenic mouse model exclusively overexpressing mutant APP (Dutch type) in neuron finally had Aβ deposited in the vessels and the positive correlation between severity of capillary CAA and neuritic pathology [7,22,23]. However, vascular origin of Aβ was also important in the formation

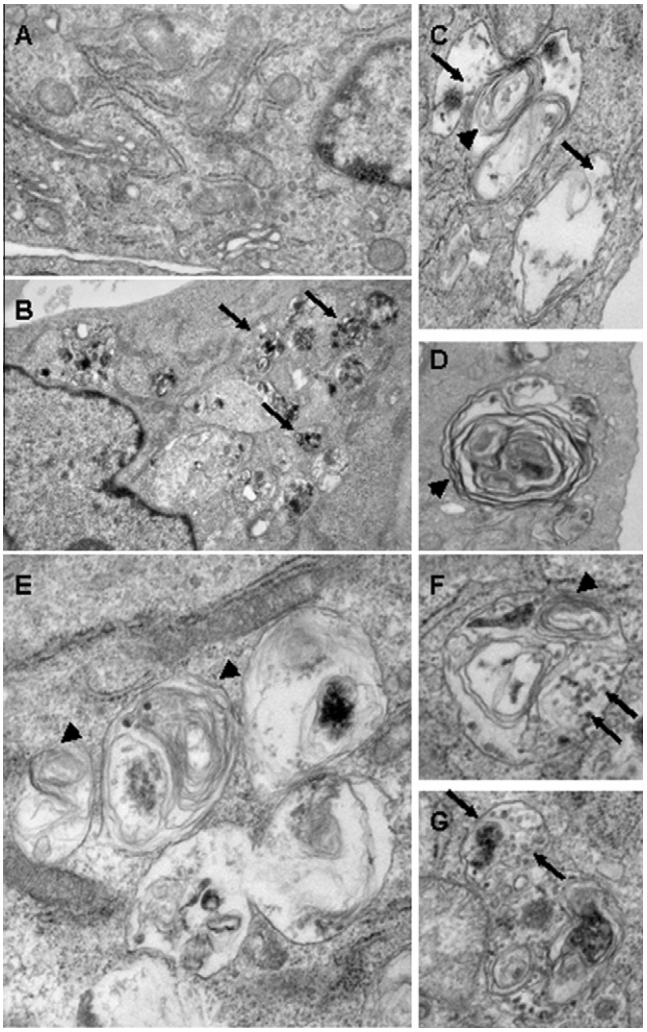

Fig. 3. Ultrastructure of autophagic vacuole in primary human umbilical vein cells after starvation. AV was rarely seen in control HUVEC (A). Starvation treatment revealed multiple autophagic compartments in cell bodies (B–G), including autophagosome containing multiple smaller compartments (B, C, F, G; arrow) or multilamellar structures (C–F; arrowhead).

of CAA and thought acting as seeds to facilitate the aggregation of Aβ and formation of CAA [24]. Here we firstly demonstrated that endothelium was capable of generating $A\beta_{42}$ under circumstance of starvation and contribute to the formation of CAA.

Endothelium is one of the important continents of brain blood barrier (BBB) which cleared Aβ that drainage from the brain [18,25]. Recently, it was suggested that BBB disruption played an important role in the chronic neurodegenerative diseases including AD [5]. Although $A\beta_{40}$ is the main continent of CAA, it was shown that $A\beta_{40}$ itself alone was not sufficient to form CAA and $A\beta_{42}$ served as seeds for aggregation of $A\beta_{40}$ drainage to the vessels [24]. However, $A\beta_{42}$ is more easily to aggregate than $A\beta_{40}$, so how $A\beta_{42}$ drainage from neurons and serve as seeds nearby vessels was a question. One answer is that vessel itself might produce $A\beta_{42}$ to act as seeds. This was important for the CAA formation because the local secreted $A\beta_{42}$ could act as seeds to aggregate less fibrillogenic $A\beta_{40}$ secreted nearby or drainage to the vessels. Accordingly, Coma et al. demonstrated that vascular smooth muscle cell (VSMC) could produce $A\beta_{42}$ and contribute to the formation of CAA [26]. Our work has addressed the contribution of endothelium to the CAA.

Starvation could cause energy deficiency which might lead to amyloid accumulation in Alzheimer's animal models [12,13]. We provide the first data that starvation could also facilitate producing $A\beta_{42}$ from endothelium. The next question is how starvation contributes to the amyloid production in endothelium. Our results showed that BACE1 and two of γ-secretase–APH and PEN2 increased in HUVEC after starvation, indicating that production of $A\beta_{42}$ might be resulted in part from increased β-cleavage and γ-cleavage of APP under starvation. We further demonstrated that starvation also increased the β-secretase activity in HUVEC. Accordingly, VSMC could generate $A\beta_{42}$ by increasing BACE1 expression in Coma's study [26]. Similarly, starvation could increase the expression of BACE1 and γ-secretase in vitro and transgenic AD animal models [12,13]. However, APP and βCTF (fragment cleaved from APP by β-secretase) levels were found constantly expressed after starvation in neurons [13,27]. This is different from our observation and indicated that different mechanisms might facilitate amyloidogenic process between neurons and HUVEC we adopted here. Davies et al. found β-secretase activity was not presented in HUVEC which was different from our study that β-secretase activity was increased under starvation. The possible explanation was that starvation might increased the comparable low level of β-secretase activity in HUVEC measured by ELISA method [28]. We should be cautious with the change of β-secretase activity under starvation and further study was needed to confirm our results.

We also found that starvation-induced macroautophagy during the process of generating $A\beta_{42}$ in HUVEC. Macroautophagy is a process involved in degrading long-lived proteins and organelle in the cell. In AD patients and APP/PS1 transgenic mouse model, autophagic vacuoles were a prominent pathological phenomenon and macroautophagy was already disturbed both in neuron and endothelium early before Aβ deposit [13]. Suppressing basal macroautophagy could cause neurodegeneration without any pathogenic insults [29], suggesting the important role of macroautophagy in the neurodegenerative diseases.

Our study showed that both suppressing macroautophagy and inhibiting autophagosome-lysosome fusion could enhance $A\beta_{42}$ production in HUVEC under starvation, indicating that macroautophagy might act to prevent the production of $A\beta_{42}$ in endothelium under starvation. This is different from Yu's study that macroautophagy facilitated $A\beta_{42}$ production in neuron [13]. Actually, many evidences suggested that macroautophagy has a dual role and the exact effect of autophagy is depended on different condition such as specific process, cell type and the level of activation [30–34]. For instance, although macroautophagy was proved to triggered Aβ release in neurons, it was also shown to prevent protein aggregation in Huntington disease and Aβ-induced neurotoxicity by increased degrading toxic proteins like Huntington and Aβ [35–38]. This change was further confirmed by the data from Pickford's study that knock-down of macroautophagic protein-beclin-1 increased the amyloid-laden with autophagic vacuoles in APP transgenic animal model and overexpression of beclin-1 could reduce extracellular amyloid accumulation [27]. Li et al. found that macroautophagy was protective from normal aging while exerted harmful effect when $A\beta_{42}$ was overexpressed [33,39,40]. In muscle cell, it was also shown that amyloid was degraded through macroautophagy pathway and suppressing it would lead to amyloid accumulation and toxicity [41–43]. Our data again demonstrated the dual role of macroautophagy in the amyloid processing in different types of cell and different condition.

In summary, we found that starvation could trigger the amyloidogenic process and lead to $A\beta_{42}$ accumulation in HUVEC. This was perhaps mediated by elevating the expression of BACE1, APH and PEN2, increasing the cleavage of APP into $A\beta_{42}$ fragments. Macroautophagy was enhanced under starvation, possibly in order to prevent $A\beta_{42}$ production under starvation. In all, our work demonstrated that endothelium was capable of secreting $A\beta_{42}$ and contributed to the CAA under starvation.

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